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Improved Detection of 3'-Hydroxystanozolol Using 3'-Hydroxystanozolol-d_3 as Internal Standard

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Introduction

3'-Hydroxystanozolol is one of the main urinary metabolites of the anabolic androgenic steroid stanozolol and is mainly excreted in a conjugated form [1]. In doping control present testing methods on stanozolol metabolites predominantly rely on gas chromatography-mass spectrometry (GC/MS). For an improved gas chromatographical behaviour the stanozolol metabolites are analysed as trimethylsilyl (TMS) derivatives, leading to sharper peaks and lower limits of detection. According to the minimum required performance limits (MRPL) of the WADA, 3'-hydroxystanozolol has to be detected at a concentration level of 2 ng/ml of urine [2]. To achieve this detection limit, high-resolution mass spectrometry (HRMS) and tandem-mass spectrometry (MSMS) have been applied. In lower concentration ranges the gas chromatographic analysis of 3'-hydroxystanozolol is very difficult. It shows a high sensitivity to active sites or disturbing matrix compounds. Because of its pyrazole structure the steroid can form bonds with any active sites in the gas chromatograph (injector, column head and cold points, e.g. in the transfer line), resulting in decreasing intensities of the corresponding signal. In this study the application of 3'-hydroxystanozolol-d_3 as an internal standard in anabolic steroid screening is presented. The effects of this deuterated standard are investigated particularly with regard to the detection of 3'-hydroxystanozolol by means of GC/HRMS and GC/tandem-MS.

Experimental

The GC/MS experiments were performed on an Agilent 6890N gas chromatograph interfaced to a Finnigan GCQ ion trap mass spectrometer or a Finnigan MAT95 high resolution mass spectrometer, respectively. Conditions were as follows: column: Ultra1 capillary column; film thickness 0.11µm, 15.9 m x 0.2 mm I.D.; carrier gas: Helium, const. pressure 13 psi, split 1:10 (1:20); injector temperature: 300°C; temperature program: 185°C, + 5°C/min to 240°C, + 20°C/min to 310°C (2 min); interface temperature 300°C; ionization: electron impact (EI)

(70 eV). The main MS instrumental conditions for the detection of 3'-hydroxystanozolol are indicated in Table 1.

Tandem-MS	HRMS
Precursor ion: m/z 545	MID Mode
Excitation voltage (V): 1.7	Ion traces : m/z 560, 545, 520
Product ions: m/z 455, 387	Resolution 5000
Ion source temperature: 225°C	Ion source temperature: 240°C

Tab.1 MS parameters for screening on 3'-hydroxystanozolol.

Sample preparation

Five different blank urines have been the base for the investigation of the carrier properties of 3'-hydroxystanozolol-d_3. For each urine different samples are prepared as follows: a blank, a reference urine containing 2 ng/ml 3'-hydroxystanozolol and four samples, also containing 2 ng/ml of 3'-hydroxystanozolol, but additionally different concentrations of 3'-hydroxy-stanozolol-d_3 (5, 10, 20, 40 ng/ml). As internal standard 17a-methyltestosterone was used. The samples were prepared according to the standard operating procedure for anabolic steroids described by Geyer et al [3]. After enzymatically cleavage of the glucuronides at pH 7, the steroids were extracted with TBME at pH 9.6. After centrifugation the organic layer is transferred and evaporated to dryness. The dry steroid residues were derivatized with 100 µl of MSTFA/NH_4I/ Ethanethiol 1000:2:3.

Results and Discussion

In contrast to most of the other AAS, the response of 3'-hydroxystanozolol strongly depends on the number of active sites in the chromatographic system and is reduced with increasing number of injections. In Figure 1 the response values of 3'-hydroxystanozolol and the metandienone metabolite epimetendiol (EMD) in the control urines (containing 2 ng /ml of each compound) exemplarily are plotted against the age of the GC column (GC/tandem-MS data). Whereas the response of 3'-hydroxystanozolol strongly decreases with increasing number of injections the EMD response is more or less constant up to at least 1600 injections. After this time the signal to noise ratio of 3'-hydroxystanozolol is becoming so low, that a replacement of the GC column is necessary.

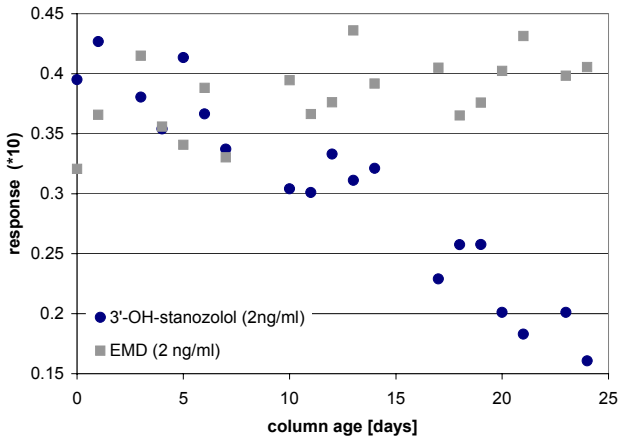

Fig.1: Response of 3'-hydroxystanozolol and epimetendiol (EMD) in control urines (approximately 1600 injections, GC/tandem-MS data).

To improve the detection of 3'-hydroxystanozolol in the screening, especially at concentrations near the MRPL, 3'-hydroxystanozolol-d_3 was used as an internal standard. Due to its same chemical behaviour like 3'-hydroxystanozolol this partial deuterated standard, if added in excess, probably saturates most of the active sites and acts as carrier for the coeluting 3'-hydroxystanozolol. Additionally it is a powerful tool for controlling the retention time as well as for the calculation of the 3'-hydroxystanozolol concentration in any suspicious sample.

Fig. 2 shows the response dependence of 2 ng 3'-hydroxystanozolol /ml in five different urines on various concentrations of the partial deuterated standard. To eliminate any carry over effects of 3'-hydroxystanozolol, leading to wrong response values, a blank is analysed before analysing every sample. In comparison to the reference value moderate carrier concentrations of 10 ng/ml result in an increased response and improved signal to noise ratio of 3'-hydroxystanozolol.

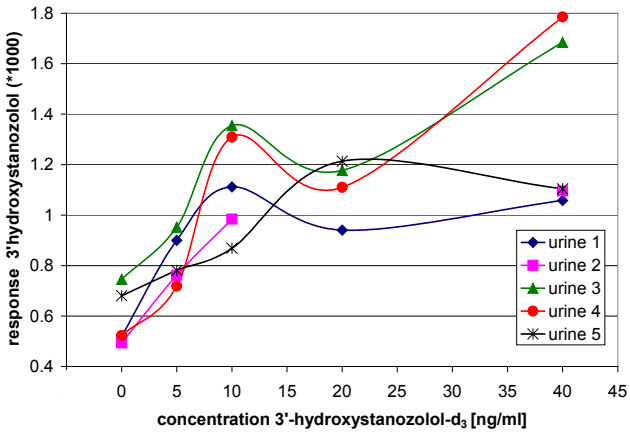

Fig. 2: Response of 3'-hydroxystanozolol dependent on the concentration of 3'-hydroxystanozolol-d_3 (GC/HRMS data).

To investigate the influence of 3'-hydroxystanozolol itself on the active sites in the GC system, the reference urine was injected repeatedly. In Fig. 3a the response changes of 3'-hydroxystanozolol in urine 1 are plotted against the number of injections. The data

exemplarily show only a slight increase of the response for 3'-hydroxystanozolol whereas the response shift for the same urine containing various carrier concentrations is significantly higher (Fig. 3b).

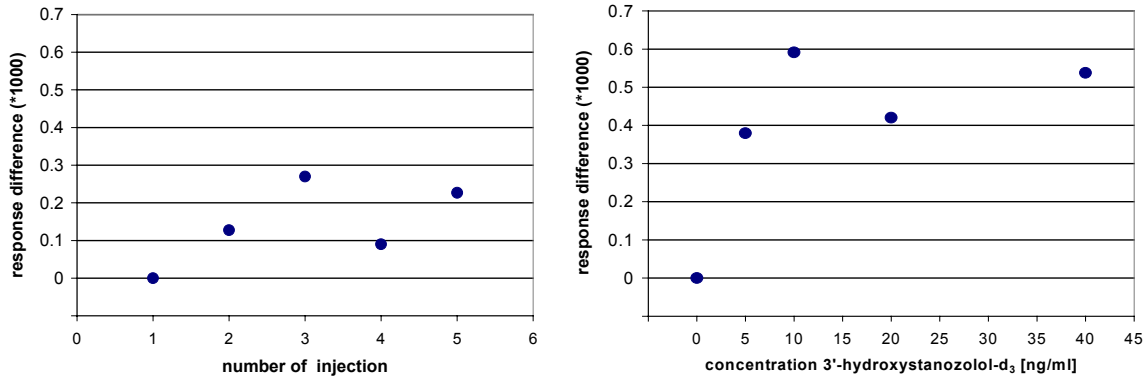

Fig.3: Response changes of 3'-hydroxystanozolol in urine 1:

a) obtained by repeated injection of the reference urine (GC/HRMS data).

b) obtained by different concentrations of 3'-hydroxystanozolol-d_3 (GC/HRMS data).

Conclusion

The internal standard 3'-hydroxystanozolol-d_3 saturates active sites in the GC system and acts as a carrier resulting in an improved detection of 3'-hydroxystanozolol. Based on this improvements the lifetime of the GC column will be prolonged. The shown data are only a snapshot for the actual state of the GC system. As the number of active sites can vary from day to day, the response changes may be different, too. Additionally the deuterated standard is a powerful tool for the calculation of the 3'-hydroxystanozolol concentration in any suspicious sample.

References

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